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(54) **PROCEDES POUR DETERMINER LA CAPACITE DES ANTAGONISTES A INFLUER SUR L'ACTIVITE DE RECEPTEURS COUPLES A DES PROTEINES G**

(54) **METHODS OF TESTING ANTAGONISTS FOR THEIR ABILITIES TO AFFECT THE ACTIVITY OF G PROTEIN-COUPLED RECEPTORS**

(57) L'invention concerne des procédés pour classer des substances et déterminer leur capacité à influer sur l'activité de récepteurs couplés à des protéines G. Plus spécifiquement, ces procédés impliquent de déterminer la capacité de l'antagoniste à augmenter l'activité spontanée de récepteurs couplés à des protéines G et à sensibiliser des récepteurs couplés à des protéines G à des agonistes. Ces procédés seront utiles dans l'industrie pharmaceutique pour le criblage de nouveaux médicaments en fonction de leur capacité à interagir avec des récepteurs couplés à des protéines G. Les réactifs nécessaires à l'utilisation desdits procédés peuvent être compris dans un kit d'analyse.

(57) Methods to test and rank substances for their abilities to affect G protein-coupled receptor activity. Specifically, these methods include testing the ability of the antagonist to increase spontaneous G protein-coupled receptor activity and to sensitive G protein-coupled receptors to agonists are described. These methods will be useful in the pharmaceutical industry for screening new drugs for their abilities to interact with G protein-coupled receptors. Reagents necessary to use this method can be supplied as part of a test kit.

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(54) Title: METHODS OF TESTING ANTAGONISTS FOR THEIR ABILITIES TO AFFECT THE ACTIVITY OF G PROTEIN-COUPLED RECEPTORS

(57) Abstract

Methods to test and rank substances for their abilities to affect G protein-coupled receptor activity. Specifically, these methods include testing the ability of the antagonist to increase spontaneous G protein-coupled receptor activity and to sensitive G protein-coupled receptors to agonists are described. These methods will be useful in the pharmaceutical industry for screening new drugs for their abilities to interact with G protein-coupled receptors. Reagents necessary to use this method can be supplied as part of a test kit.

METHODS OF TESTING ANTAGONISTS FOR THEIR ABILITIES TO AFFECT THE ACTIVITY OF G PROTEIN-COUPLED RECEPTORS

FIELD OF THE INVENTION

The present invention relates to methods of screening test substances for their abilities to interact with and modulate the functional properties of G protein-coupled receptors. More specifically, the present invention describes methods of testing and ranking substances for their abilities to increase spontaneous G protein-coupled receptor activity and to sensitize G protein-coupled receptors to agonists.

BACKGROUND OF THE INVENTION

G protein-coupled receptors (GPCRs) are proteins located in the plasma membrane of cells. They function as one part of a multi-component complex involved in signal transmission. GPCRs share a common signaling mechanism, whereby signal transduction across the membrane involves intracellular transducer elements known as G proteins (named for their ability to bind and hydrolyze the nucleotide GTP). When a chemical messenger binds to a specific site on the extracellular surface of the receptor, the conformation of the receptor changes so that it can interact with and activate an intracellular G protein. This causes guanosine diphosphate (GDP), which is bound to the surface of the G protein, to be replaced by guanosine triphosphate (GTP), triggering another conformational change in the G protein. Once GTP is bound to its surface, the G protein regulates the activity of an effector. These effectors include enzymes such as adenylyl cyclase and phospholipase C, ion channels that are specific for calcium ions (Ca^{2+}), potassium ions (K^+), or sodium ions (Na^+), and certain transport proteins.

In general, activation of GPCRs by transmitters will induce one of the following effector responses: activation of adenylyl cyclase, inhibition of adenylyl cyclase, or stimulation of phospholipase C activity. When the effector adenylyl cyclase is either activated or inhibited, it produces changes in the concentration of the molecule cyclic adenosine monophosphate (cAMP). Another effector, phospholipase C, causes one molecule of phosphatidylinositol-bisphosphate (PIP_2) to be cleaved into one molecule each of inositol triphosphate (IP_3) and diacylglycerol (DAG); IP_3 then causes calcium ions (Ca^{2+}) to be released into the cytoplasm. cAMP and Ca^{2+} are termed second messengers. Alterations in cellular levels of second messengers act to alter the behavior of other target proteins in the cell.

GPCRs activate a number of different types of signaling pathways in cells. This activation occurs at the level of the G proteins, which detect and direct signals from diverse receptors to the appropriate effector-response pathway. Although there are many forms of G proteins, the three main forms are as follows: Gs-like, which mediate activation of adenylyl cyclase; Gi-like, which mediate inhibition of adenylyl cyclase; and Gq-like, which mediate activation of phosphoplipase C. Since one receptor can activate many G proteins, the signal can be greatly amplified through this signal transduction pathway.

A wide variety of chemical messengers, involved in regulating key functions in the body, act through GPCRs. These include neurotransmitters such as dopamine, acetylcholine, and serotonin, hormones of the endocrine system such as somatostatin, glucagon, and adrenocorticotropin, lipid mediators such as prostaglandins and leukotrienes, and immunomodulatory proteins such as interleukin-8 and monocyte-chemoattractant polypeptide. The family of GPCRs also includes the receptors for light (rhodopsin), for odors (olfactory receptors) and for taste (gustatory receptors). Over one hundred different GPCRs have been identified in humans, and many more are expected to be discovered. Most of these receptors are believed to utilize one of the three principal G protein-effector

signaling pathways (stimulation or inhibition of adenylyl cyclase (AC) or activation of phospholipase C (PLC)). Examples of neurotransmitter GPCRs and their respective effector signaling pathways are shown in Table 1.

Table 1. Examples of Neurotransmitter GPCRs

	Effector Signaling Pathway		
Neurotransmitter	**Stimulates AC**	**Inhibits AC**	**Stimulates PLC**
Acetylcholine		m_2, m_4	m_1, m_3, m_5
Adenosine		A_1, A_2	
Corticotropin-Releasing Factor		CRF-R	
Cannabinoids		R[c]	
Dopamine	D_1, D_5	D_2	
Histamine	H_2		
Neuropeptide Y		Y_1, Y_2, Y_3	
Norepinephrine, epinephrine	$_2$-AR $_1$-AR	$_2$-AR	
Opioids		μ	
Serotonin	5-HT_4	5-HT_{1A}, 5-HT_{1B}, 5-HT_{1D}	5-HT_2

A number of compounds can bind to GPCRs. These compounds are called ligands. Analysis of the effects of ligands on the ability of GPCRs to activate effector signaling pathways suggests that the receptors exist in two different conformations. an inactive, silent conformation and an active conformation that triggers G protein activation and effector signaling (Gilman, A.G., 1987, *Annu. Rev, Biochem.* 56: 615-649; Levitski, A., 1988, *Science* 241:800-806). An increasing amount of evidence suggests that GPCRs isomerize rapidly between the active and inactive forms.

Ligands that bind to the transmitter recognition site on the receptor may be classified into two types: 1) agonists, which mimic the action of natural transmitters and activate GPCRs; and 2) antagonists, which bind to the receptors but do not activate the CPR. Agonists cause the receptor to assume the active conformation. The amplitude of activation of GPCRs by agonists is termed efficacy and is an important parameter in assessing the therapeutic potential of a drug: a full agonist elicits a maximal response in a given tissue or system while a partial agonist elicits only a fraction of the maximal response at full occupancy of the GPCR.

Antagonists prevent the activation of receptors. Until recently, it was generally believed that antagonists act by binding to the receptor and blocking agonist binding, without having any effect on receptor activity. It has now become apparent that antagonists can also act by turning off spontaneously-active GPCRs (GPCRs that are active even in the absence of agonist) (Schütz and Freissmuth (1992) *Trends Pharmacol. Sci.* 13:376-380.). These negative regulators are called inverse agonists. Antagonists also exhibit efficacy: full antagonists completely turn off receptor activity while partial antagonists only partially inhibit this activity. Thus, antagonists are now thought of as negative regulators, *i.e.* They turn receptors off in two ways: by blocking agonist action and by inhibiting spontaneous receptor activity.

This evidence has led to a model in which active and inactive receptors co-exist in the cell in equilibrium, with agonists pushing the equilibrium to the active form, inverse agonists pushing it to the inactive form, and neutral antagonists blocking the agonist action without favoring either conformation.

Effects of agonists and antagonists described above are acute effects, *i.e.* represent effects of drug on receptor activity when the drug (agonist or antagonist) is bound to the receptor. However, the effects of both agonists and antagonists on GPCR activity can change following chronic exposure to these drugs.

A well-characterized example of chronic effect is agonist-induced receptor desensitization of the β_2-adrenergic receptor. A decrease in the efficacy of an agonist is noted following an extended exposure of receptor to agonist, when removal of agonist is followed by immediate measuring of maximal receptor response to the agonist. The observed decrease in maximal response upon second agonist challenge is referred to as desensitization and is believed to occur through a combination of different mechanisms, including removal of the receptor from the cell surface (sequestration) and enzymatic phosphorylation of the activated receptor such that it is unable to interact with G protein transducers. This phenomenon has a negative impact on therapies, since the efficacy of the agonist drug decreases with time. As tolerance to the drug increases, the dose of drug must be increased to maintain the therapeutic effect.

In some cases, chronic treatment with antagonist drugs has been reported to produce an increase in the number of GPCRs (e.g. dopamine antagonists in animal models). In other cases, however, chronic treatment with antagonists have been shown to promote decreases in the number of GPCRs (e.g. $5HT_1$ receptors and serotonergic antagonists; see Meltzer and Nash (1991) *Pharmacol. Rev.* 43:588-600.). In particular, it has been shown that antagonists can decrease ligand binding capacity of the rat 5-hydroxytryptamine Type 2C ($5HT_{2C}$) receptor

(Labrecque *et al.*, (1995) *Mol. Pharm.* 48:150-159.). This decrease in GPCR binding sites following antagonist treatment has been termed "atypical down-regulation". It is thought that the down-regulation of GPCRs is a distinct action from inverse agonist activity.

These effects of chronic antagonist treatment have not been associated previously with changes in receptor response to subsequent agonist treatment. However, acute antagonist effects have been described which suggest that antagonists may have effects on receptor responsiveness. A property termed "augmentative antagonism" has been described in tissue studies, on a very few occasions whereby the simultaneous addition of an antagonist and an agonist to the receptor causes an increase in the maximal response to the agonist. This observation has been interpreted as evidence that certain antagonists may increase the ability of the receptor to activate G proteins in response to agonists.

In view of the diverse effects of GPCRs in the human body, it is not surprising that the pharmaceutical sector has great interest in the development of new drugs to target GPCRs. These drugs have potential as therapeutic agents in a wide range of human pathologies, including psychiatric disorders (depression, psychoses, bipolar disorder), metabolic disorders (diabetes, obesity, anorexia nervosa), cancer, autoimmune disorders, cardiovascular disorders, neurodegenerative disorders (e.g. Alzheimer's disease), and pain disorders.

There are no methods of testing antagonists as drug candidates on the basis of their ability to effect changes in activities of GPCRs. Specifically, while there is a limited body of evidence suggesting that antagonists may promote an increase in receptor response to agonists in certain tissue preparations, there exists no method to systematically rank antagonists for their relative abilities to exert this effect. Moreover, there is no known method of comparing antagonists for their relative abilities to increase spontaneous receptor activity, since this is a property of antagonists which has not been previously described.

Thus, in determining whether particular antagonists are candidates for suitable drug treatments, there is a need for methods of testing these substances for their ability to affect the activities of GPCRs. It is therefore an object of the present invention to provide a method of analyzing test substances for two manners of affecting receptor activities: 1) for their abilities to increase the response of GPCRs to agonists, and 2) to increase spontaneous GPCR activity.

This background information is provided for the purpose of making known information believed by the applicant to be of possible relevance to the present invention. No admission is necessarily intended, nor should be construed, that any of the preceding information constitutes prior art against the present invention. Publications referred to throughout the specification are hereby incorporated by reference in their entireties in this application.

SUMMARY OF THE INVENTION

To meet the needs noted above, the present invention describes methods for testing and comparing antagonists for their abilities to affect GPCR activities, that exploits two new newly recognized abilities of antagonists: 1) to increase the response of GPCRs to agonists, and 2) to increase spontaneous GPCR activity. These methods will have direct application to the screening of antagonist drugs for potential therapeutic utility, and will be particularly useful since the sensitizing effect of antagonist drugs is ligand selective.

These methods are applicable to any recombinant GPCRs that can be expressed in heterologous host cells.

In one embodiment, the method of the present invention involves the following: 1) expressing cloned cDNA encoding a GPCR in a heterologous host cell; 2) treating these cells with different concentrations of test substance; 3) washing the

cells to remove the test substance; and 4) measuring the ability of the receptors to activate cellular response pathways in the absence of added agonist (spontaneous receptor activity).

In another embodiment, the method of the present invention involves the following: 1) expressing cloned cDNA encoding a GPCR in a heterologous host cell; 2) treating these cells with different concentrations of test substance; 3) washing the cells to remove the test substance; and 4) measuring the ability of the receptors to activate cellular response pathways in the presence of increasing concentrations of agonist drug (agonist response).

In yet another embodiment, this invention involves a test kit including whole cells or membrane fragments or cell membrane preparation which can express cloned GPCRs, appropriate reagents, and supporting documentation enabling the working of this invention.

A further embodiment of the present invention involves a method of screening test substances for their abilities to increase spontaneous G protein-coupled receptor activity, comprising:

a. expressing DNA encoding a G protein-coupled receptor in a cell expression system;

b. contacting a test substance with the cells under conditions permitting interaction of the test substance with the expressed G protein-coupled receptor;

c. washing the cells under appropriate conditions to remove the test substance; and

d. measuring a quantifiable parameter using biochemical or other assay procedures that indicate the agonist-independent activity of said receptor in said system.

Yet a further embodiment of the present invention involves a method of screening test substances for their abilities to increase the response of G protein-coupled receptors to agonists, comprising: (1) expressing DNA encoding a G protein-

coupled receptor in a cell expression system; (2) contacting a test substance with the cells under conditions permitting interaction of the test substance with the expressed G protein-coupled receptor; (3)washing the cells under appropriate conditions to remove the test substance; (4) contacting an agonist with the cells under conditions permitting interaction of the agonist with the expressed G protein-coupled receptor; and (5) measuring a quantifiable parameter using biochemical or other assay procedures that indicate the agonist-dependent activity of said receptor in said system.

Still yet a further embodiment of the present invention involves a test kit for screening test substances for their abilities to increase G protein-coupled receptor activity, in one or more containers, comprising: a) whole cells expressing a G protein-coupled receptor; b) appropriate reagents and buffers to allow the contacting of test substances with said receptor; c) appropriate reagents and buffers to enable the washing of said cells to remove the test substances; d) appropriate reagents, buffers, biochemical assay components to enable detection and/or quantification of activity of G protein-coupled receptors; and e) instructions for use of this kit.

Still yet a further embodiment of the present invention involves a test kit for screening test substances for their abilities to increase spontaneous G protein-coupled receptor activity, in one or more containers, comprising: a) whole cells expressing a G protein-coupled receptor; b) appropriate reagents and buffers to allow the contacting of test substances with said receptor; c) appropriate reagents and buffers to enable the washing of said cells to remove the test substances; d) appropriate reagents, buffers, and agonists to permit the interaction of such agonist with said receptor; e) appropriate reagents, buffers, biochemical assay components to enable detection and/or quantification of activity of G protein-coupled receptors; and f) instructions for use of this kit.

Still yet a further embodiment of the present invention involves a test kit for screening test substances for their abilities to affect the agonist response of G protein-coupled receptor activity, in one or more containers, comprising: a) whole cells expressing a G protein-coupled receptor; b) appropriate reagents and buffers to allow the contacting of test substances with said receptor; c) appropriate reagents and buffers to enable the washing of said cells to remove the test substances; d) appropriate reagents, buffers, biochemical assay components to enable detection and/or quantification of activity of G protein-coupled receptors; and e) instructions for use of this kit.

Still yet a further embodiment of the present invention involves a test kit for screening test substances for their abilities to affect the agonist response of G protein-coupled receptor activity, in one or more containers, comprising: a) whole cells expressing a G protein-coupled receptor; b) appropriate reagents and buffers to allow the contacting of test substances with said receptor; c) appropriate reagents and buffers to enable the washing of said cells to remove the test substances; d) appropriate reagents, buffers, and agonists to permit the interaction of such agonist with said receptor; e) appropriate reagents, buffers, biochemical assay components to enable detection and/or quantification of activity of G protein-coupled receptors; and f) instructions for use of this kit.

Still yet a further embodiment of the present invention involves an *in vitro* method to evaluate a G protein-coupled receptor antagonist, the test compound, for the ability to increase spontaneous G protein-coupled receptor activity of its corresponding G protein-coupled receptor, the method comprising: (a) incubating the test compound for a limited period of time with a whole cell or cell membrane preparation comprising a cloned and expressed G protein-coupled receptor, washing out any residual test compound prior to evaluating its effects; and (b) measuring the extent to which the test compound.

Still yet a further embodiment of the present invention involves an *in vitro* method to evaluate a G protein-coupled receptor antagonist, the test compound, for the ability to increase the spontaneous receptor activity or effect on agonist response of its corresponding G protein-coupled receptor, the method comprising: (a) using biochemical or other assay procedures to measure a quantifiable parameter that is indicative of the density of receptors ability to activate a cellular response pathway in a whole cell or cell membrane preparation, comprising a cloned and expressed G protein-coupled receptor; (b) incubating the test compound with an aliquot of the preparation for a limited period of time; (c) washing out residual test compound from the preparation; and (d) measuring the quantifiable parameter in the test compound-treated aliquot to determine the extent to which the test compound increases the spontaneous receptor activity or effects the agonist response of the receptor.

Still yet a further embodiment of the present invention involves an *in vitro* method to evaluate a G protein-coupled receptor antagonist, the test compound, for the ability to increase the spontaneous receptor activity or effect on agonist response of its corresponding G protein-coupled receptor, the method comprising the steps, to be followed sequentially, of: (a) generating a reproducible expression system that allows for discrimination of chemical compounds based on their relative ability to increase the spontaneous receptor activity or effect on agonist response of said G protein-coupled receptors wherein said system expresses a gene coding for a G protein-coupled receptor in a cultured cell-line in a manner that causes biologically active receptors to become incorporated into the cell membrane at a concentration that is sufficiently high that it permits ranking of the potencies of the abilities of antagonists to increase the spontaneous receptor activity or effect on agonist response on a statistically significant basis, yet not so high as to induce cell toxicity;(b) generating a cell preparation or cell membrane preparation from the expression system; (c) measuring a quantifiable parameter that is indicative of the G protein-coupled receptors; (d) contacting an aliquot taken from the preparation with a test compound under conditions permitting interaction of the

test compound with the G protein-coupled receptor;(e)washing out residual test compound from the aliquot; and (f) measuring said quantifiable parameter in the aliquot subsequent to (e) to determine the extent to which said compound to increase the spontaneous receptor activity or effect on agonist response of said receptor.

Various other objects and advantages of the present invention will become apparent from the detailed description of the invention.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1. Effects of serotonergic antagonist pretreatment on IP release in $5HT_{2C}$-expressing Sf9 cells. Sf9 cells labeled with [^{3}H]myo-inositol (1 μCi/ml) were infected 48 hours with a baculovirus encoding the $5HT_{2C}$ receptor and treated for 60 min. with various concentrations of methysergide (A), mianserin (B), metergoline (C), clozapine (D), loxapine (E), and chlorpromazine (F) as indicated on the abscissa. Treated and control cells were washed extensively in parallel, then incubated in the presence of 1 μM 5HT (white circle), 1 μM mianserin (white square), or vehicle (black triangle). IP levels were measured in whole cells in this representative experiment where the point shown represents averaged untransformed data from a triplicate assay. The half maximal estimates of the increase in receptor function from 3 independent drug treatments are as follows: metergoline 3.8 ± 0.7 nM; mianserin 1.9 ± 0.7 μM; and clozapine 4.9 ± 1.7 μM.

Figure 2. 5HT and mianserin dose response curves after different concentrations of metergoline pretreatment on $5HT_{2C}$ receptors expressed in Sf9 cells. These results demonstrate effects of agonist on IP release in $5HT_{2C}$-expressing Sf9 cells after various pretreatments with metergoline. Sf9 cells labeled with [^{3}H]myo-inositol were infected with the $5HT_{2C}$ baculovirus. At 48 hours after infection, cells were pre-treated for 60 min. with either vehicle (control black square and dotted curve) or various concentrations of metergoline (5M - white square; 6M -

black circle; 7M - black lozenge; 8M - inverted black triangle; 9M - upright black triangle) before the washout protocol was performed. IP levels were then measured in pre-treated whole cells in the presence of various concentrations of 5HT (panel A) or mianserin in panel B as indicated on the abscissa. Data are from a representative experiment of triplicate assays; averaged results from three independent experiments (EC_{50}) are reported in Table 2.

Figure 3. $5HT_{2C}$ receptors expressed in Sf9 cells. Receptor activity after different antagonist pretreatments on Sf9 cells expressing the rat $5HT_{2C}$ receptor demonstrating effects of serotonergic antagonists on apparent loss of [^{3}H]mesulergine binding sites in $5HT_{2C}$-containing Sf9 membranes following antagonist pretreatment. Cells were prepared at 48 hours post-infection from Sf9 cells infected with the $5HT_{2C}$ baculovirus, then treated with the indicated $5HT_{2C}$ antagonists for 1 hour. Treated and control membranes were washed extensively in parallel, then incubated with [^{3}H]mesulergine (10 nM) to determine the residual binding after drug treatment. For each ligand, data from three independent experiments were analyzed simultaneously assuming a common value for EC_{50} (see Table 3), with $Y_{(X-0)}$ and $Y_{(X-\infty)}$ unconstrained. The curves and averaged points (n=3) shown were derived from a representative experiment obtained on the same day with all drugs tested in parallel on the same membranes. Values from a representative set of data were scaled taking the fitted values of $Y_{(X-0)}$ as 100%, and the points shown represent (the averages of) the scaled data (± SEM). Each curve was generated using the fitted values of EC_{50}, with $Y_{(X-0)}$ set to 100% and $Y_{(X-\infty)}$ set to the average of $[((Y_{(X-\infty)} / Y_{(X-0)}) * 100\%]$. For clarity, points corresponding to concentration < (EC_{50}/10000) were omitted from the figure. Values for EC_{50} are reported in Table 2.

DETAILED DESCRIPTION OF THE INVENTION

The following terms and abbreviations are used throughout the specification and in the claims:

A "agonist response" is defined as the ability of CPCRs to activate cellular response pathways: (a) in the presence of agonist, or (b) in response to agonist.

A "chemical-messenger" is defined as any messenger, in the absolute broadest sense, natural or unnatural, that induces an effect or blocks an effect on a proteinaceous receptor, including chemical substance transmitters (e.g. neurotransmitters, hormones, and lipid mediators such as prostaglandins and leukotrienes; usually act on chemoreceptors), light (e.g. signaling via a rhodopsin receptor; usually acts on electromagnetic (photo) receptors), and stimuli such as temperature and mechanical signals (e.g. physical or chemical damage, vibration, touch, pressure, movement) which act on mechanoreceptors, thermoreceptors, and nociceptors.

An "effector protein" is defined as any protein that is activated or inactivated by a G protein. Some examples include adenylyl cyclase and phospholipase C.

A "G protein" is defined as any member of the family of signal transducing guanine nucleotide binding proteins.

A "G protein-coupled receptor" is defined to be any cell surface transmembrane protein that, when activated by a chemical, mediates signal transduction by coupling with a heterotrimeric guanine nucleotide-binding protein (G protein).

A "ligand" is intended to include any substance that interacts with a receptor. It may simulate, inhibit, or cause some effect for the activity of the receptor. An

"agonist" is defined as a ligand increasing the functional activity of a receptor (ie. signal transduction through the receptor). A "neutral antagonist" is defined as a ligand that can bind to the transmitter recognition site on the receptor and thereby block receptor activation by agonists. An "inverse agonist" is defined as a ligand that can decrease the spontaneous activity of the receptor.

A "receptor" is intended to include any molecule present inside or on the surface of a cell, which can affect cellular physiology when either stimulated or inhibited by a ligand.

A "second messenger" is defined as an intermediate compound whose concentration, either intercellularly or within the surrounding cell membrane, is raised or lowered as a consequence of the activity of an effector protein. Some examples of second messengers include cyclic adenosine monophosphate (cAMP), phophatidylinositol (PI), calcium ions (Ca^{+2}), and arachidonic acid derivatives.

A "spontaneous receptor activity" is defined as the ability of CPCRs to activate cellular response pathways in the absence of agonist.

A "test substance" is intended to include any drug, compound, or molecule with potential biological activity.

The present invention relates to methods of testing substances for their abilities to increase the response of GPCRs to agonists and to increase spontaneous GPCR activity.

These methods are based on the discovery that certain antagonists can differentially increase the efficacy of agonists or the spontaneous activity of GPCRs. These sensitizing effects represent novel activities of antagonists which

may involve improving receptor coupling efficiency and reducing receptor density.

Antagonist induced sensitization of GPCRs is likely to be of significance in the treatment of disease since it could be used to revert a lack of agonist responsiveness to a normal responsive level. It could also be used if poor coupling of agonist to the GPCR is implicated in the etiology of disease. Specific therapeutic applications of drugs that re-sensitize GPCRs could be important novel paradigms for drug development. These alternative methods of antagonist action should be considered when exploring therapeutic processes involving antagonist drugs.

Creation of Recombinant Vector and Infection of Cell Lines:

The preliminary step of the assay method of the present invention is the expression of GPCRs in heterologous host cells. For clarity, the $5HT_{2C}$ receptor is used as an example; however, the descriptions apply equally well to any GPCR whose cDNA has been cloned, such as thyrotropin, lutropin-choriogonadotropin, dopamine, and histamine receptors. Likewise, once cDNAs become available for other GPCRs, they can also be used in the present invention as described below. This method is applicable to any GPCR once a cDNA clone for that receptor has been generated.

The cDNA coding for functional GPCR can be inserted into any suitable vector, cloned, and expressed in an appropriate cell line. An appropriate cell line is one that will be able to express and process the receptor, in addition to possessing the necessary biochemical machinery to respond to signal transduction through a given receptor. Transfection may be performed according to known methods. In general, a cDNA sequence encoding a receptor may conveniently be subjected to recombinant DNA procedures.

The vector may be an autonomously replicating vector, such as a plasmid, or it may be one which, when introduced into a host cell, is integrated into the host cell genome and replicated together with the chromosome(s) into which it has been integrated. Using procedures that are well known to those skilled in the art, DNA expression vectors incorporating coding regions for the receptor will be suitable for replication in the appropriate host cell. Eukaryotic viral vectors such as insect, and mammalian viral vectors can be constructed using vectors such as the baculovirus, the Sindbis virus, the Semliki Forest virus or the vaccinia virus. Retroviral and adenoviral vectors can also be used. The DNA sequence encoding the receptor should be operably connected to a suitable promoter sequence and a suitable terminator sequence, and may further comprise a DNA sequence enabling the vector to replicate in the host cell in question. The procedures used to ligate the DNA sequences coding for the receptor, the promoter, and the terminator, and to insert them into suitable vectors containing the information necessary for replication are well known to persons skilled in the art (Sambrook *et al.*, (1989) *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Laboratory, Cold Spring Harbor, New York.).

Cells that may be used in the present method are cells that are able to express the cloned receptor at a sufficiently high level, in addition to possessing the ability to activate signal transduction in cell signal pathways. Such cells are typically eukaryotic cells, such as mammalian or insect cells. Methods of transfecting mammalian cells and expressing DNA sequences introduced in the cells are well known to persons skilled in the art (Kaufman and Sharp (1982) *J. Mol. Biol.* 159:601-621; Southern and Berg (1982) *J. Mol. Appl. Genet.* 1:327-341; Loyter *et al.*, (1982) *Proc. Natl. Acad. Sci. USA* 79:422-426; Wigler *et al.*, (1978) *Cell* 14:725; Corsaro and Pearson (1981) *Somatic Cell Genetics* 7:603; Graham and van der Eb (1973) *Virology* 52:456; Neumann *et al.*, (1982) *EMBO J.* 1:841-845; and Wigler *et al.*, (1977) *Cell* 11:223-232.).

One such vector and expression system that works particularly well with this invention entails constructing a recombinant baculovirus expression vector, capable of expressing a GPCR in a host insect cell line (e.g. Sf9 cells). Examples of how to construct suitable recombinant baculovirus vectors are described in U.S. Patents No. 4,745,051 and 4,879,236. The general procedures of recombinant DNA technology pertaining to creation and manipulation of the baculovirus system are well known to those skilled in the art (O'Reilly *et al.*, (1992) *Baculovirus Expression Vectors, a Laboratory Manual* (New York: W.H. Freeman and Company); Davies (1994) *Bio/Technology* 12:47-50.).

One embodiment of this invention describes and demonstrates the expression of GPCRs in a baculovirus-insect cell system. The *Autographa californica* nuclear polyhedrosis baculovirus (AcNPV) has been shown to be suitable as a viral expression vector for the efficient production in cultured insect Sf9 cells of mammalian membrane proteins from foreign genes (Luckow and Summers (1988) *Bio/Technology* 6:47-55; Miller (1988) *Annu. Rev. Microbiol.* 42:177-199.). A number of GPCRs have been expressed in the baculovirus-Sf9 cell expression system, and were found to maintain binding properties characteristic of the "natural" receptors in tissues or expressed from cloned cDNAs in mammalian cell lines (Mouillac *et al.*, (1992) *J. Biol. Chem.* 267:21733-21737; Parker (1991) *Biol. Chem.* 266:519-527; Wong (1990) *J. Biol. Chem.* 265:6219-6224.).

One particular embodiment of the invention involves $5HT_{2c}$ receptors that are expressed within a baculovirus expression system in Sf9 cells. It has been shown that the infection of Sf9 cells with recombinant baculovirus encoding the $5HT_{2c}$ receptor leads to the expression of functional receptors capable of regulating intracellular levels of IPs (Labrecque *et al.*, (1995) *supra*). The levels of $5HT_{2c}$ receptor expression in the Sf9/baculovirus system are roughly 20-fold higher than those of the native receptor in rat choroid plexus and more than five-fold higher than the levels reported for NIH/3T3 cells expressing the recombinant receptor. As observed with mammalian systems expressing either native or recombinant

$5HT_{2C}$ receptors, the baculovirus-expressed $5HT_{2C}$ receptor activates polyphosphoinositide hydrolysis in response to serotonergic agonists via a pertussis toxin-insensitive pathway. The expressed receptor exhibits spontaneous activation of inositol phosphate production, which is inhibited in a dose-dependent manner by serotonergic antagonists, consistent with inverse agonist activity.

Screening of Test Substances:

Once a functional GPCR expression system is obtained, test substances can be screened to determine whether they increase spontaneous GPCR activity or increase GPCR response to agonists. This involves treating the recombinant cells with different concentrations of test substance, then washing the cells to remove the test substance. The ability of the GPCRs to activate cellular response pathways in the absence of added agonist (spontaneous receptor activity) or in the presence of increasing concentrations of agonist drug (agonist response) is then measured. Activation of cellular response pathways can be measured by any suitable quantifiable parameter using biochemical or other assay procedures that indicate the activity of the cloned receptor in the expression system. Many appropriate biochemical and other assay procedures using standard techniques are well known to persons skilled in the art. One example of such an assay involves determining the activity of effectors such as adenylyl cyclase or phospholipase C. The activity of the effector is determined by measuring levels of second messenger such as cAMP (for example, Gilman (1970) *Proc. Natl. Acad. Sci. USA* 67:305-312.) or inositol phosphate (Fargin *et al.*, (1989) *J. Biol. Chem.* 254:14818-14852.).

In a preferred embodiment of this invention, Sf9 cells infected with the $5HT_{2C}$/baculovirus vector are pre-treated with varying concentrations of different test substances. After extensive washing of residual test substance, assays are performed on whole cell preparations to determine the effect of these substances on the spontaneous activity of GPCRs and on the response of receptors to agonists. This is achieved by measuring inositol phosphate levels.

The present invention is described in further detail in the following non-limiting examples. It is to be understood that the examples described below are not meant to limit the scope of the present invention. It is expected that numerous variants will be obvious to the person skilled in the art to which the present invention pertains, without any departure from the spirit of the present invention. The appended claims, properly construed, form the only limitation upon the scope of the present invention.

EXAMPLE I

Reagents:

Buffer chemicals and protease inhibitors were purchased from Sigma, and cell culture media from Gibco/BRL. Unlabeled ligands were supplied by Research Biochemicals International, with the exception of Loxapine, which was a gift from Dr. B. Roth. [^{3}H] mesulergine (78-82 Ci/mM) was purchased from Amersham and [^{3}H]myo-inositol (10-20 Ci/mM) was purchased from NEN-Dupont. The AG 1 X 8 ion exchange resin was supplied by Bio-Rad.

Construction of Recombinant Baculovirus:

The recombinant baculovirus used for expression of the rat $5HT_{2C}$ receptor was provided by the Biotechnology Research Institute of Montreal. The virus was constructed using a synthetic DNA fragment encoding the rat $5HT_{2C}$ receptor, based on the published sequence of the cloned cDNA from choroid plexus (Julius *et al.*, (1988) *Science* 241:558-564.). The synthetic cDNA was prepared and its sequence verified by Allelix Biopharmaceuticals (Mississauga, ON). The cDNA was inserted into the IpDC-126 baculovirus transfer vector and a recombinant baculovirus was produced and purified as previously described (O'Reilly *et al.*, (1992) *Baculovirus Expression Vectors: A Laboratory Manual*).

Cell Culture and Receptor Expression:

Sf9 cells were cultured in 50 ml batches in 250 ml shaker flasks at 27°C in Sf-900 II serum free medium containing 50 µg/ml gentamicin sulfate. Cells were grown to a density of 3 X 10^6 cells/ml and infected with the $5HT_{2c}$ recombinant baculovirus or with wild-type *Autographia californica* nuclear polyhedrosis baculovirus, at a multiplicity of infection of 2. Viral stocks for infections were in Grace's insect medium containing 5% fetal bovine serum (Hyclone) and were added to cultures at a dilution of approximately 1/20 upon infection. The infected cells were maintained in culture for various periods and used for analysis of ligand binding and measurement of IPS production as described below.

Radioligand Binding Assays:

For the estimation of total receptor numbers in intact Sf9 cells, the cells were pelleted by low-speed centrifugation (3 min. at 800 rpm in Sorvall H6000A rotor), followed by resuspension in PBS and re-centrifugation, and resuspended in either PBS or binding buffer (50 nM Tris/HCl, pH 7.4, 15 mM $MgCl_2$, 2 mM EDTA, 0.1% ascorbic acid, 5 mg/ml leupeptine, 10 mg/ml aprotinin, 20 mg/ml benzamidine, 50 mg/ml TPCK, and 50 mg/ml trypsin inhibitor). Cell viability after washing was estimated at 60-70% by trypsan blue exclusion. Aliquots of 10,000 cells were incubated for 1 hour at 27°C in a final volume of 540 ml containing 20 nM of [^{3}H]mesulergine. Incubations were terminated by vacuum filtration over GF/C filters and washing with binding buffer at 4°C. Bound radioactivity was measured on filters impregnated with MeltiLex™ melt-on scintillant using a Wallac MicroBeta counter. Nonspecific binding was estimated in parallel incubations containing 10mM mianserin or metergoline.

For analysis of ligand binding to membrane preparations, cultures were harvested at 48 hr post-infection, lysed, and a membrane pellet was prepared as previously described (Labrecque *et al.*, (1992) *FEBS letters* 304:157-162.). These were stored at -80°C. Protein concentrations were determined by the nitrocellulose amido black method (Schaffner and Weissmann (1973) *Analytic. Biochem.* 56:502-551.). Membranes were thawed on ice and resuspended in binding buffer

by homogenization in a Potter homogenizer. The membranes (5 mg) were incubated with [^{3}H]mesulergine for 1 hour at 27°C in a final volume of 540 ml, and the assays terminated as described above for intact cells. Saturation binding assays yielded a Kd for [^{3}H]mesulergine of 2 nM, and competition binding assays with unlabeled drugs were carried out with 3 nM [^{3}H]mesulergine (Table 1).

Inositol Phosphate Production:

Growing cell cultures (1 X 10^6 cells/ml) were prelabeled with 2 mCi/ml [^{3}H]myo-inositol for 24 hours prior to infection, and the labeled cells were then transferred to 50 ml shaker flasks for infection. At 48 hours after infection, Sf9 cell cultures were treated with antagonists by adding drugs directly to the culture medium 1 hour before cell harvest. Cells from treated cultures were extensively washed in culture medium (4 X 1h) to remove unbound ligands and resuspended for polyinositolphosphate production as described in Labrecque *et al.*, (1995) *Mol. Pharm.* 48:150-159.

Effect of Drug Pretreatment on [^{3}H]mesulergine Binding:

Growing cell cultures (1 X 10^6 cells/ml) were prelabeled for 24 hours with 2 mCi/ml [^{3}H]myo-inositol prior to infection, and the labeled cells were then transferred to 50 ml shaker flasks for infection. At 48 hours after infection, Sf9 cell cultures were treated with antagonists by adding drugs directly to the culture medium 1 hour before cell harvest. Cells from treated cultures were extensively washed in culture medium (4 X 1h) to remove unbound ligands. and resuspended for polyinositolphosphate production and for parallel binding with 10 nM [^{3}H]mesulergine as described (Labrecque *et al.*, (1995) *Mol. Pharm.* 48:150-159. Further details are described in Table and Figure legends.

Analysis of Data:

The binding of [^{3}H]mesulergine (saturation experiments) and the inhibition of [^{3}H]mesulergine binding by unlabeled serotonergic ligands were analyzed in terms of a single class of binding site using the computer program LIGAND (Munson

and Rodbard (1980) *Analytic. Biochem.* 107:220-239.). Data from three independent binding experiments were fitted individually and the affinities presented for [^{3}H]mesulergine (Kd) and other ligands (Ki) represent as the average values (± SEM) from 3 sets of data. Dose-response data for Ips and antagonist-induced decreases in [^{3}H]mesulergine binding capacity were scaled taking values measured in the absence of added ligand as 100%. The scaled data were analyzed according to a four parameter logistic equation analogous to the Hill equation (ALLFIT: A. DeLean, Department of Pharmacology, Université de Montréal, or INPLOT: Prizm Software, San Diego, CA). For the decrease in [^{3}H]mesulergine binding with each ligand, three to four sets of data were fitted simultaneously using ALLFIT with the slope factor set equal to 1. Further details are described in Tables and Figure legends. Maximal inverse agonist activities measured for each drug tested were compared statistically by a two tailed t test (a=0.05).

Expression of functional $5HT_{2C}$ receptors in Sf9 Cells:

A baculovirus encoding the rat 5HT2C receptor was used to express the recombinant receptor in cultures of Sf9 insect cells. Receptor levels, as measured in whole cells by the binding of [3H]mesulergine (10nM), increased with time after infection to reach approximately 1 X 10^6 sites/cell at 72 hours after infection (data not shown).

Analysis of Antagonist Pretreatment on Inositol Phosphate Activity:

Six serotonergic antagonists were selected to be screened for their abilities to affect GPCR activities: metergoline, mianserin, methysergide, clozapine, loxapine, and chlorpromazine. All six are known to demonstrate good affinities for the rat $5HT_{2C}$ receptor (Roth *et al.* (1991) *J. Pharmacol. Exp. Ther.* 260:1362-1365). Cells were pretreated with varying concentrations of the antagonists, then washed extensively. Cells were then incubated, either unstimulated to measure spontaneous activity, or induced by agonist. The cells were then assayed for production of inositol phosphate (IP). Results are shown in Figure 1.

Increased Spontaneous Activity of GPCRs:

Of the six pretreatments performed, metergoline, mianserin, and the atypical neuroleptic clozapine had significant effects on the level of spontaneous receptor activity as compared to control vehicle pretreated cells. These spectacular and unexpected increases in spontaneous activity were concentration dependent. These same three drugs inhibit spontaneous receptor activity and display inverse agonist effect in acute assays (Labrecque *et al.*, (1995) *supra*). In contrast, pretreatment with methysergide, loxapine, and chlorpromazine were unable to change the spontaneous activity of the $5HT_{2C}$ receptor.

Antagonist pretreatment regiments were performed across a range of concentrations varying from vehicle to 100 μM. The IP activity demonstrates a byphasic response curve for the sensitizing effect of metergoline, mianserin, and clozapine pretreatment on the spontaneous ligand-independent activity of the receptor. The unusual bell shape curve suggests that spontaneous receptor activation increased up to a peak level with increasing concentration of antagonist treatment, but past a threshold of antagonist concentration, levels of spontaneous receptor activation returned to a lower basal level.

These observations suggest that specific antagonist pretreatments have direct effects on the basal level of spontaneous activity of GPCRs and a concomitant effect on the level of GPCR binding sites. The variation in receptor density observed would seem to be implicated in this complex alteration of spontaneous levels of receptor activation.

Increased GPCR Response to Agonists:

Serotonin efficacy is greatly increased following pretreatments with metergoline, mianserin, or clozapine (Fig. 1). This increase is concentration dependent. The pretreatment regimens show a byphasic response curve for agonist response, similar to the bell shaped curve corresponding to elevated spontaneous activity. Both curves display superimposable ascending components resulting in equivalent

rank order potency (EC_{50}) for the sensitizing phenomenon induced by increasing concentration of antagonist pretreatment. The descending component of the basal unstimulated curves drop at a concentration inferior to the same component of the agonist response curves.

The resulting elevation in 5HT efficacy represents a phenomenal increase in agonist response determined by the absolute level of sensitization and by the level of spontaneous activity present in the system. The results indicate that lower concentrations of pretreatment have greater impact on spontaneous levels of activation than on the actual agonist efficacy.

The maximal agonist response observed after antagonist sensitization treatment indicates that the rank order for the magnitude of this effect was metergoline (350% ± 125) > mianserin (300% ± 125) > clozapine (60% ± 20) ≥ loxapine (40%) ≥ chlorpromazine (30% ± 35) ≥ methysergide (20% ± 20). This ranking of maximal sensitizing efficacy of antagonist pretreatment is different than their corresponding rank order potency for the increased 5HT response described above. Although estimated maximal percent IP stimulation with 1 µM 5HT after antagonist pretreatment varied between experiments, the extent of the stimulation observed was always within 300% to 350% over basal level for metergoline and mianserin pretreatment. These stimulation levels correspond accurately to levels of maximal 5HT stimulation observed in choroid plexus (400%) (Sanders-Bush and Breeding (1989) *J. Pharmacol.Exp. Ther.* 252:984-988; Sanders-Bush and Breeding (1988) *J. Pharmacol.Exp. Ther.* 247:169-173.), or in NIH/3T3 cells (200%) (Barker *et al.*, (1994) *J. Biol. Chem.* 269:11887-11890.).

Figure 2 shows a series of dose response curves with 5HT following antagonist (metergoline) pretreatment at various concentrations. Figure 2A reports the effect of metergoline treatment on 5HT dose-response production of IPs. The results indicate that metergoline pretreatment at concentrations inferior to 10 nM produce

5HT dose responses identical to vehicle treated cells. In contrast, higher concentrations of metergoline have significant effects on 5HT dose response.

Cell pretreatment with 10 nM metergoline displayed a large increase in basal IP levels suggesting an increase in spontaneous receptor activation. A ten-fold increase in metergoline concentration to 100 nM significantly reduced the basal IP levels without significant effect on maximal level. Further increases in metergoline to concentrations superior to 100 nM progressively and significantly decreased 5HT potency (EC_{50}) and reduced the maximal response of IPs produced in the presence of 5HT (Fig. 3A, Table 2).

Table 2. Pharmacology of 5HT2C receptors expressed in Sf9 cells after metergoline treatment.

(Results are expressed as a mean ± SE for at least 3 experiments. IP responses and [^{3}H]mesulergine binding are measured in whole cells (48 hours post-infection) following pretreatment with metergoline at various concentrations.)

	Density of [^{3}H]mesulergine Binding Sites	5HT Dose Response			Maximum Activity[1]
Metergoline Pretreatments	% Residual Sites	-pEC$_{50}$ ± SE	p values[2]	% Stimulation[3]	Magnitude
vehicle	100 ± 10	7.69 ± 0.10		12 ± 2	0.03
-9M	57 ± 14	7.33 ± 0.06	>0.05	13 ± 2	0.00
-8M	29 ± 12	7.06 ± 0.02	>0.05	39 ± 5	0.94
-7M	11 ± 4	7.07 ± 0.08	0.03	112 ± 4	1.00
-6M	6 ± 4	6.42 ± 0.12	0.01	129 ± 3	0.87
-5M	5 ± 4	5.40 ± 0.09	0.01	70 ± 4	0.22

[1]Maximal IP levels from Figure 2 were normalized to determine the maximal efficacity following treatment of the cells with metergoline.

[2]Student t test was performed between vehicle treated and the various metergoline treatments (a=0.05).

[3]Values estimated from Figure 2.

From the foregoing description, one skilled in the art can easily ascertain the essential characteristics of this invention, and without departing from the spirit and scope thereof, can make various changes and modifications to the invention to adapt it to various usages and conditions. Such changes and modifications are properly, equitably, and intended to be within the full range of equivalence of the following claims.

We Claim:

1. A method of screening test substances for their abilities to increase spontaneous G protein-coupled receptor activity, comprising:

   a. expressing DNA encoding a G protein-coupled receptor in a cell expression system;

   b. contacting a test substance with the cells under conditions permitting interaction of the test substance with the expressed G protein-coupled receptor;

   c. washing the cells under appropriate conditions to remove the test substance; and

   d. measuring a quantifiable parameter using biochemical or other assay procedures that indicate the agonist-independent activity of said receptor in said system.

2. A method according to claim 1, wherein whole cells are derived from a baculovirus expression system in Sf9 cells.

3. A method according to claims 1 or 2, wherein said receptor is a 5-hydroxytryptamine Type 2C receptor.

4. A method of screening test substances for their abilities to increase the response of G protein-coupled receptors to agonists, comprising:

   (1) expressing DNA encoding a G protein-coupled receptor in a cell expression system;

(2) contacting a test substance with the cells under conditions permitting interaction of the test substance with the expressed G protein-coupled receptor;

(3) washing the cells under appropriate conditions to remove the test substance;

(4) contacting an agonist with the cells under conditions permitting interaction of the agonist with the expressed G protein-coupled receptor; and

(5) measuring a quantifiable parameter using biochemical or other assay procedures that indicate the agonist-dependent activity of said receptor in said system.

5. A method according to claim 4, wherein whole cells are derived from a baculovirus expression system in Sf9 cells.

6. A method according to claims 4 or 5, wherein said receptor is a 5-hydroxytryptamine Type 2C receptor.

7. A method according to claims 4, 5, or 6, wherein said agonist is 5-hydroxytryptamine.

8. A test kit for screening test substances for their abilities to increase G protein-coupled receptor activity, in one or more containers, comprising:

a) whole cells expressing a G protein-coupled receptor;

b) appropriate reagents and buffers to allow the contacting of test substances with said receptor;

c) appropriate reagents and buffers to enable the washing of said cells to remove the test substances;

d) appropriate reagents, buffers, biochemical assay components to enable detection and/or quantification of activity of G protein-coupled receptors; and

e) instructions for use of this kit.

9. A test kit for screening test substances for their abilities to increase spontaneous G protein-coupled receptor activity, in one or more containers, comprising:

a) whole cells expressing a G protein-coupled receptor;

b) appropriate reagents and buffers to allow the contacting of test substances with said receptor;

c) appropriate reagents and buffers to enable the washing of said cells to remove the test substances;

d) appropriate reagents, buffers, and agonists to permit the interaction of such agonist with said receptor;

e) appropriate reagents, buffers, biochemical assay components to enable detection and/or quantification of activity of G protein-coupled receptors; and

f) instructions for use of this kit.

10. A test kit for screening test substances for their abilities to affect the agonist response of G protein-coupled receptor activity, in one or more containers, comprising:

a) whole cells expressing a G protein-coupled receptor;

b) appropriate reagents and buffers to allow the contacting of test substances with said receptor;

c) appropriate reagents and buffers to enable the washing of said cells to remove the test substances;

d) appropriate reagents, buffers, biochemical assay components to enable detection and/or quantification of activity of G protein-coupled receptors; and

e) instructions for use of this kit.

11. A test kit for screening test substances for their abilities to affect the agonist response of G protein-coupled receptor activity, in one or more containers, comprising:

a) whole cells expressing a G protein-coupled receptor;

b) appropriate reagents and buffers to allow the contacting of test substances with said receptor;

c) appropriate reagents and buffers to enable the washing of said cells to remove the test substances;

d) appropriate reagents, buffers, and agonists to permit the interaction of such agonist with said receptor;

e) appropriate reagents, buffers, biochemical assay components to enable detection and/or quantification of activity of G protein-coupled receptors; and

f) instructions for use of this kit.

12. An *in vitro* method to evaluate a G protein-coupled receptor antagonist, the test compound, for the ability to increase spontaneous G protein-coupled receptor activity of its corresponding G protein-coupled receptor, the method comprising:

(a) incubating the test compound for a limited period of time with a whole cell or cell membrane preparation comprising a cloned and expressed G protein-coupled receptor, washing out any residual test compound prior to evaluating its effects; and

(b) measuring the extent to which the test compound.

13. The method as in claim 12, wherein said whole cell is an Sf9 cell or said membrane fragment is derived thereof.

14. The method as in claim 12, wherein said G protein-coupled receptor is $5HT_{2C}$.

15. The method as in claim 12, wherein said G protein-coupled receptor is a serotonin receptor selected from the group consisting of $5HT_{1A}$, $5HT_{1B}$, $5HT_{1D}$, HT_{1E}, $5HT_{1F}$, $5HT_{2A}$, $5HT_{2B}$, $5HT_{4}$, $5HT_{5A}$, $5HT_{5B}$, $5HT_{6}$, $5HT_{7}$.

16. An *in vitro* method to evaluate a G protein-coupled receptor antagonist, the test compound, for the ability to increase the spontaneous receptor

activity or effect on agonist response of its corresponding G protein-coupled receptor, the method comprising:

(a) using biochemical or other assay procedures to measure a quantifiable parameter that is indicative of the level of receptors ability to activate a cellular response pathway in a whole cell or cell membrane preparation, comprising a cloned and expressed G protein-coupled receptor;

(b) incubating the test compound with an aliquot of the preparation for a limited period of time;

(c) washing out residual test compound from the preparation; and

(d) measuring the quantifiable parameter in the test compound-treated aliquot to determine the extent to which the test compound increases the spontaneous receptor activity or effects the agonist response of the receptor.

17. The method as in claim 16, wherein said whole cell is an Sf9 cell or said membrane fragment is derived thereof.

18. The method as in claim 16, wherein said G protein-coupled receptor is $5HT_{2C}$.

19. The method as in claim 16, wherein said G protein-coupled receptor is a serotonin receptor selected from the group consisting of $5HT_{1A}$, $5HT_{1B}$, $5HT_{1D}$, HT_{1E}, $5HT_{1F}$, $5HT_{2A}$, $5HT_{2B}$, $5HT_4$, $5HT_{5A}$, $5HT_{5B}$, $5HT_6$, $5HT_7$.

20. An *in vitro* method to evaluate a G protein-coupled receptor antagonist, the test compound, for the ability to increase the spontaneous receptor activity or effect on agonist response of its corresponding G protein-coupled receptor, the method comprising the steps, to be followed sequentially, of:

(a) generating a reproducible expression system that allows for discrimination of chemical compounds based on their relative ability to increase the spontaneous receptor activity or effect on agonist response of said G protein-coupled receptors wherein said system expresses a gene coding for a G protein-coupled receptor in a cultured cell-line in a manner that causes biologically active receptors to become incorporated into the cell membrane at a concentration that is sufficiently high that it permits ranking of the potencies or the abilities of antagonists to increase the spontaneous receptor activity or effect on agonist response on a statistically significant basis, yet not so high as to induce cell toxicity;

(b) generating a cell preparation or cell membrane preparation from the expression system;

(c) measuring a quantifiable parameter that is indicative of the G protein-coupled receptors;

(d) contacting an aliquot taken from the preparation with a test compound under conditions permitting interaction of the test compound with the G protein-coupled receptor;

(e) washing out residual test compound from the aliquot; and

(f) measuring said quantifiable parameter in the aliquot subsequent to (e) to determine the extent to which said compound to increase the spontaneous receptor activity or effect on agonist response of said receptor.

21. The method as in claim 20, wherein said whole cell is an Sf9 cell or said membrane fragment is derived thereof.

22. The method as in claim 20, wherein said G protein-coupled receptor is $5HT_{2C}$.

23. The method as in claim 20, wherein said G protein-coupled receptor is a serotonin receptor selected from the group consisting of $5HT_{1A}$, $5HT_{1B}$, $5HT_{1D}$, HT_{1E}, $5HT_{1F}$, $5HT_{2A}$, $5HT_{2B}$, $5HT_{4}$, $5HT_{5A}$, $5HT_{5B}$, $5HT_{6}$, $5HT_{7}$.

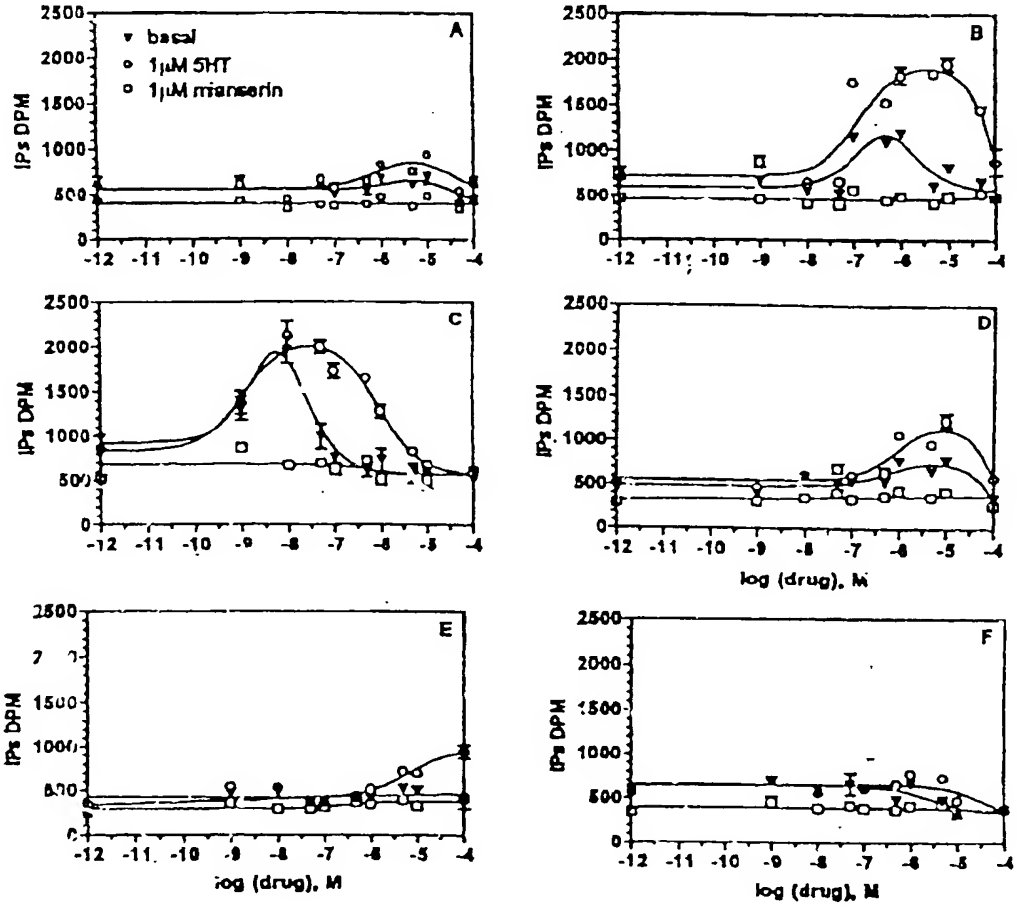

FIGURE 1

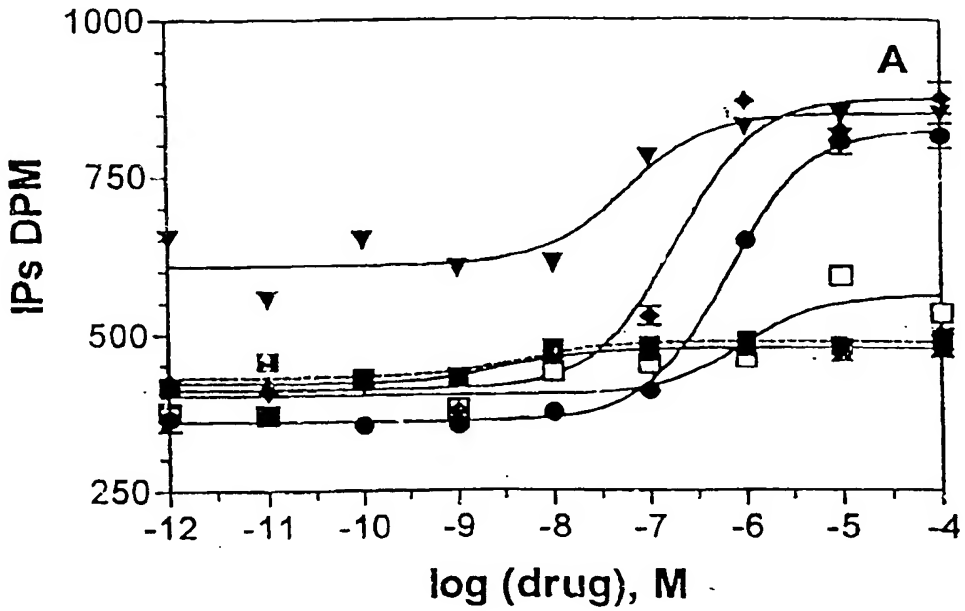

FIGURE 2A

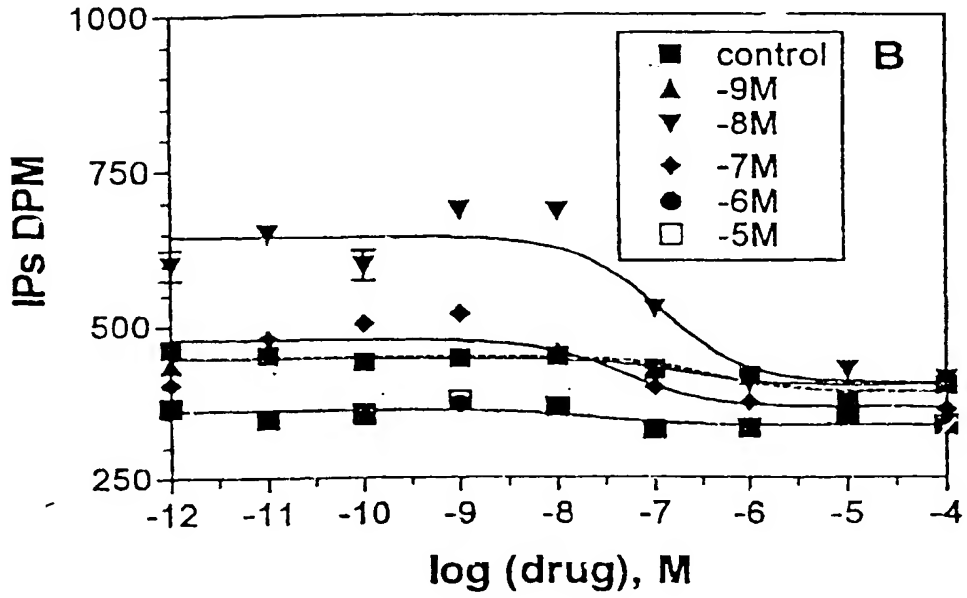
1000
750
500
250
IPs DPM
-12
-11
-10
-9
-8
-7
-6
-5
-4
log (drug), M
control
-9M
-8M
-7M
-6M
-5M
B

FIGURE 2B

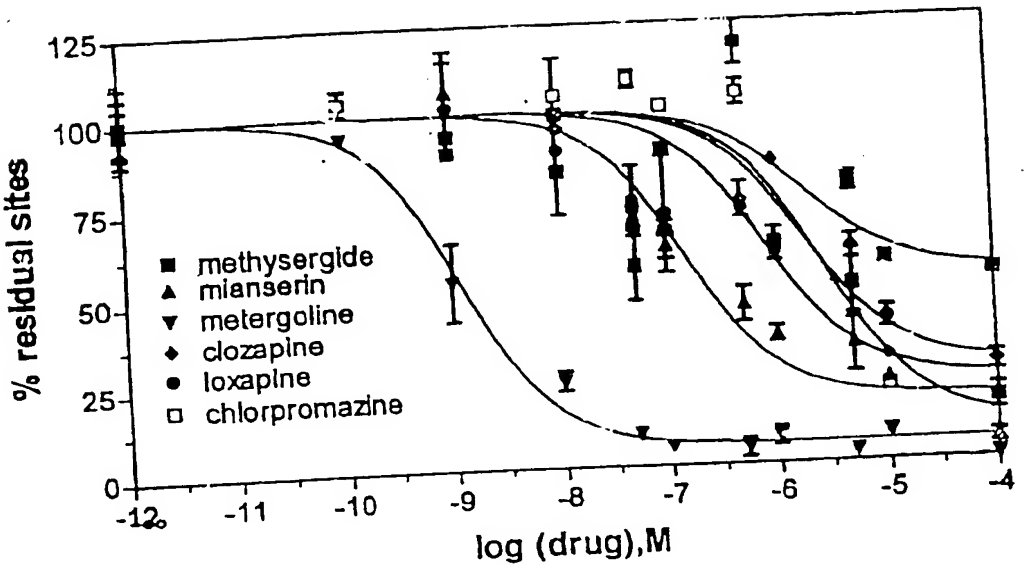
125
100
75
50
25
0
% residual sites
methysergide
mianserin
metergoline
clozapine
loxapine
chlorpromazine
-12
-11
-10
-9
-8
-7
-6
-5
-4
log (drug),M

FIGURE 3